4-2-1 Definition and classification of edible soy flours and grits

Soy flours are products obtained by finely grinding full-fat dehulled soybeans or defatted flakes made from dehulled soybeans. To be called soy flour, at least 97% of the product must pass through a 100-mesh standard screen.

Soy grits have essentially the same composition as flour, but coarser granulation. They are usually classified into three groups, according to particle size:

Coarse 10 to 20 mesh
Medium 20 to 40 mesh
Fine 40 to 80 mesh

Circle and Smith (1972) have pointed out that the name soy flour may be misleading, since its composition is totally different from that of the popular product commonly known as flour, i.e. wheat flour. They suggested alternative names such as "defatted soy solids" (as non-fat milk solids) or "soy powder" or "soy pulverate".

Edible soy flours are made from dehulled beans, hence their relatively low crude fibre and high protein content.

Soy flours (or grits) are classified according to their lipid content as follows:

* **Defatted soy flour,** obtained from solvent extracted flakes, contains less than 1% oil.

* **Full-fat soy flour**, made from unextracted,dehulled beans, contains about 18% to 20% oil.

* **Low fat soy flour,** made by adding back some oil to defatted soy flour. Lipid content varies according to specifications, usually between 4.5% and 9%.The most common range is between 5% and 6%.

* **High fat soy flour,** produced by adding back soybean oil to defatted flour, usually at the level of 15%.

* **Lecithinated soy flour**, made by adding soybean lecithin to defatted, low fat or high fat soy flours in order to increase their dispersibility and impart emulsifying properties.. Lecithin content varies according to specifications, usually up to 15%.

Commercial soy flours and grits are further classified according to their **Nitrogen Solubility Index (NSI)**, or their **Nitrogen Dispersibility Index (NDI)**. It will be recalled that these parameters indicate the extent of protein denaturation and hence the intensity of heat treatment which has been applied to the starting material. Flours made from "white flakes" have NSI values of about 80%, while those made from toasted flakes show NSI levels of 10 to 20%. Other grades are available over the entire range of intermediate NSI values.The specification of a specific value of NSI reflects , in fact, a compromise between the need to maintain the functional properties of the soy proteins or some enzyme activity, and the desire to inactivate anti-nutritional factors and eliminate the beany taste, all in function of the end use.

4.4 Defatted soy flours and grits

4-4-1 Production processes

The processes for the manufacture of raw or heated dehulled solvent extracted flakes have been described in the previous sections (Chapter 3).

Usually, all the flakes made for edible products are flash-desolventized, then carefully steam-heated to the desired NSI value.

The final milling is critical and energy consuming. Although identity standards require milling to 97% minus 100-mesh, specialty flours (such as those used as milk solids replacement in infant formulae) are ground to a finer particle size.

At such levels of fineness, the conventional hammer mill is practically useless. Impact turbo mills or high-speed pin mills have to be used.

5.3 Production processes

5-3-1 The aqueous alcohol wash process

The process is based on the ability of aqueous solutions of lower aliphatic alcohols (methanol, ethanol and isopropyl alcohol) to extract the soluble sugar fraction of defatted soy flour without solubilizing its proteins. The optimal concentration of alcohol for this process is about 60% by weight.

The theory of solvent extraction (see para. 3-2-4)is applicable to the extraction of defatted soy flour with aqueous alcohol.

Starting with defatted white flakes as raw material, the process consists of the following steps: Liquid-solid extraction, removal and recovery of the solvent from the liquid extract, removal and recovery of the solvent from the extracted flakes, drying and grinding of the flakes.

a- Solid-liquid extraction: This can be carried out batchwise or continuously. Continuous extraction is justified for relatively large scale operations. According to Campbell *et al.*(1985), continuous processes are employed for plants with typical capacities over 5,000 tons per year. Unlike oilseed crushing industries, smaller plants are not uncommon in this branch. The batch process is, therefore, rather widely applied. The methods and types of equipment used are essentially similar to those encountered in oil extraction plants: horizontal belt and basket extractors, stationary and rotary cell extractors etc. In the case of alcohol extraction, the solvents are quite volatile and flammable. Adequate precautions for the prevention of fire and explosion are necessary.

The reason for using high-NSI white flakes as the starting material is not necessarily related to the objective of obtaining a product with high protein solubility.(As explained above, this would not help anyhow , due to the different type of protein denaturation caused by the alcohol.) The principal reason for preferring this type of raw material is due to the fact that the percentage of extractable soluble sugars in white flakes is higher than in toasted meal. Toasting renders the sugars less soluble by binding them to proteins (Maillard reaction) or by caramelization. As a result of this type of condensation reactions, the sugars are no longer extractable by the solvent and they remain in the product, lowering the protein concentration in it. Furthermore, the darker colour of concentrates made from overheated meal is also objectionable, and their nutritional value is lower (lower lysine availability.)

b- Removal and recovery of the solvent from the liquid extract: The alcohols are removed from the liquid extract by evaporation and rectified by distillation. They are then brought to the proper concentration and recycled through the extractor. The distillation residue is an aqueous solution of the sugars and other solubles. It is concentrated to the consistency of honey and sold as " **soy molasses".** Typically, soy molasses contain 50% total soluble solids. These solids consist of carbohydrates (60%), proteins and other nitrogenous substances (10%), minerals (10%), fats and lipoids (20%). It is mainly used as a caloric ingredient and as a binding agent in animal feeds.

c- Desolventizing the solids: After extraction, the solvent saturated flakes are desolventized . The methods are essentially the same as for the removal of hexane from soybean meal flakes. Flash desolventizing, using superheated vapours of the alcohol-water mixture can be applied to protein concentrates. Any excess water left in the flakes after desolventizing is removed by hot air drying.

d- Grinding: The methods and equipment used to grind soy protein concentrate flakes are essentially the same as those employed in the production of soy flours (see Section 4-3-1).

8.3 Production processes

8-3-1 The traditional process

This is the method by which soymilk is made for daily consumption , particularly in China. The soybeans are washed, soaked overnight and then ground to a slurry. Cold water is added. after thorough mixing, the dilute slurry is strained through cheese cloth and pressed. The extract (soymilk) is boiled, strained again and filled into various types of containers. This is a heavy, quite gritty suspension and has a strong beany flavour and chalky mouthfeel.

8-3-2 The Soya Technology Systems (STS) Process

This is a complete technology-package making use of modern techniques of the dairy processing technology. A flow diagram of the process is shown in Fig. 36. Following is a description of the process principles, reproduced from the STS publication entitled "Soymilk in Brief" (STS 1986).

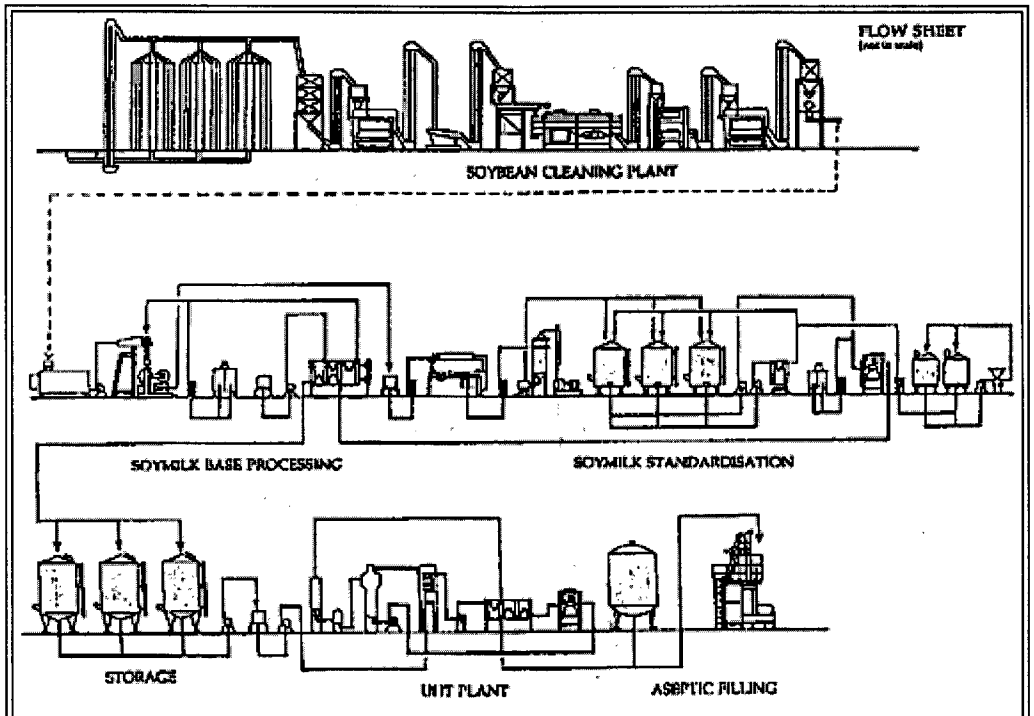


Figure 36: Pictorial Layout of SDS Soymilk Plant (Courtesy: Danish Turnkey Dairies Ltd.

"Producing a nutritious tasting soymilk for human consumption is thus considerably more complicated than just grinding the soybeans with water.

Certain factors have to be controlled during the process, such as:

- *cleaning and dehulling without damaging the soybeans*
- *destruction of the lipoxidase enzymes which cause off- flavour development.*
- *elimination of flatulence-causing oligosaccharides*
- *inactivation of the trypsin inhibitors which are present in the raw soybeans.....*

- maintenance of high-protein efficiency ratio
- removal of undesirable smell
- removal of sedimentable solids. "

Soybean Varieties:

Any organization contemplating large scale production of soymilk should make a survey of the available types of soybeans in order to select those that will give those that will give soymilk with the best flavour and colour, and the best recovery of protein, solids, and fats. These factors vary widely from one soybean variety to another. High grade beans generally produce the best soymilk, and the large-seeded soybeans are considered to be the superior type.

Storage:

Moulds and insects are the primary causes of quality deterioration in stored soybeans. Both are favoured by high moisture, warm temperatures and the presence of damaged soybeans and foreign material. This being the case, it is important that the soybeans be essentially sound, clean and dry if they are to be stored for any period of time. Moisture control is the key to successful storage.

Cleaning:

Commercial soybeans contain varying amounts of foreign material such as dirt, dust, stones etc. It is vital for the production of high quality soymilk that this foreign material be removed.

Just as important is the elimination of damaged soybeans from the supply to the soymilk processing plant. This is necessary because the enzyme lipoxidase ... will have acted on the fatty acids in the damaged soybean cell tissue, producing compounds with the characteristic beany flavour.

Dehulling:

Soybean hulls contain unwanted substances. Also the hulls are an obstruction to continued processing, especially in the decanter. Soil bacteria are present in the soybean hulls. The hulls should therefore be removed to reduce bacteria count in the soymilk, resulting in better flavour and shelf-life. Soybean hulls contain polysaccharides which should be removed to avoid off-flavours and processing problems caused by foaming. The holding time for heat treatment of the soybeans to inactivate undesired enzymes can be shortened when using dehulled soybeans. This will decrease protein denaturation and browning of the soymilk.

Dehulled soybeans produce a white, attractive, appetizing soymilk.

Blanching/Enzyme Inactivation:

Blanching of the soybeans in a solution of sodium bicarbonate at high temperature starts the inactivation of the bitter taste causing enzyme lipoxidase. This treatment also washes out water soluble oligosaccharides (flatulence causing) and starts the inactivation of trypsin inhibitor (reducing digestibility).

Grinding:

Grinding in a hot water solution of sodium bicarbonate converts the soybeans into a colloid solution

(soyslurry) without unwanted enzyme activity.

Fibre Separation (decanting):

In order to avoid chalkiness and obtain a good mouthfeel of the soymilk, the insoluble fibres are filtered away. To achieve optimal results, a decanter centrifuge is used....

Deodorization:

To remove unwanted volatile off-flavours the clarified soymilk base is deaerated by means of vacuum and parboiling in the deodorizer.

Standardization:

Water is added to the soymilk base to obtain the desired protein content of the soymilk...

Flavouring and Formulation:

One of the keys to widespread acceptance of soymilk is proper formulation, using sweetening and flavouring agents of the types and in the amounts suited to local tastes. The addition of oil to soymilk results in increased richness and creaminess (good mouthfeel) and adds calories....

Fortification:

Soymilk for school-feeding programs in developing countries is preferably fortified with vitamins and minerals, e.g. vitamin B-12 and calcium. Soymilk has only 1/5 of the calcium in cow's milk (but 1/2 of that in mother's milk).

Homogenization:

Homogenization breaks down fat globules into very fine particles by forcing them under great pressure through minute valve openings, and distributes them evenly throughout the soymilk. Otherwise the fats would tend to lump together, rise to the surface, and separate as a distinct layer. Homogenization gives soymilk a creamier, more uniform consistency.

UHT (Ultra-high temperature) Treatment:

Direct method UHT treatment of soymilk serves two purposes:

(1) to inactivate bacteria thereby prolonging shelf life.

(2) to deodorize the soymilk.....

....The UHT treated product should be aseptically packed."

As it seen from the above description, the STS process makes use of advanced dairy processing techniques, such as Ultra-high temperature (UHT) sterilization and aseptic packaging. It is not surprising, therefore, that the process has been adopted by the Danish Turnkey Dairies Ltd., a company specializing in the supply and erection of dairy and related plants on a world wide basis. Fig. 37 shows a tri-dimensional layout of a standard 4,000 litre per hour soymilk plant supplied by the company.

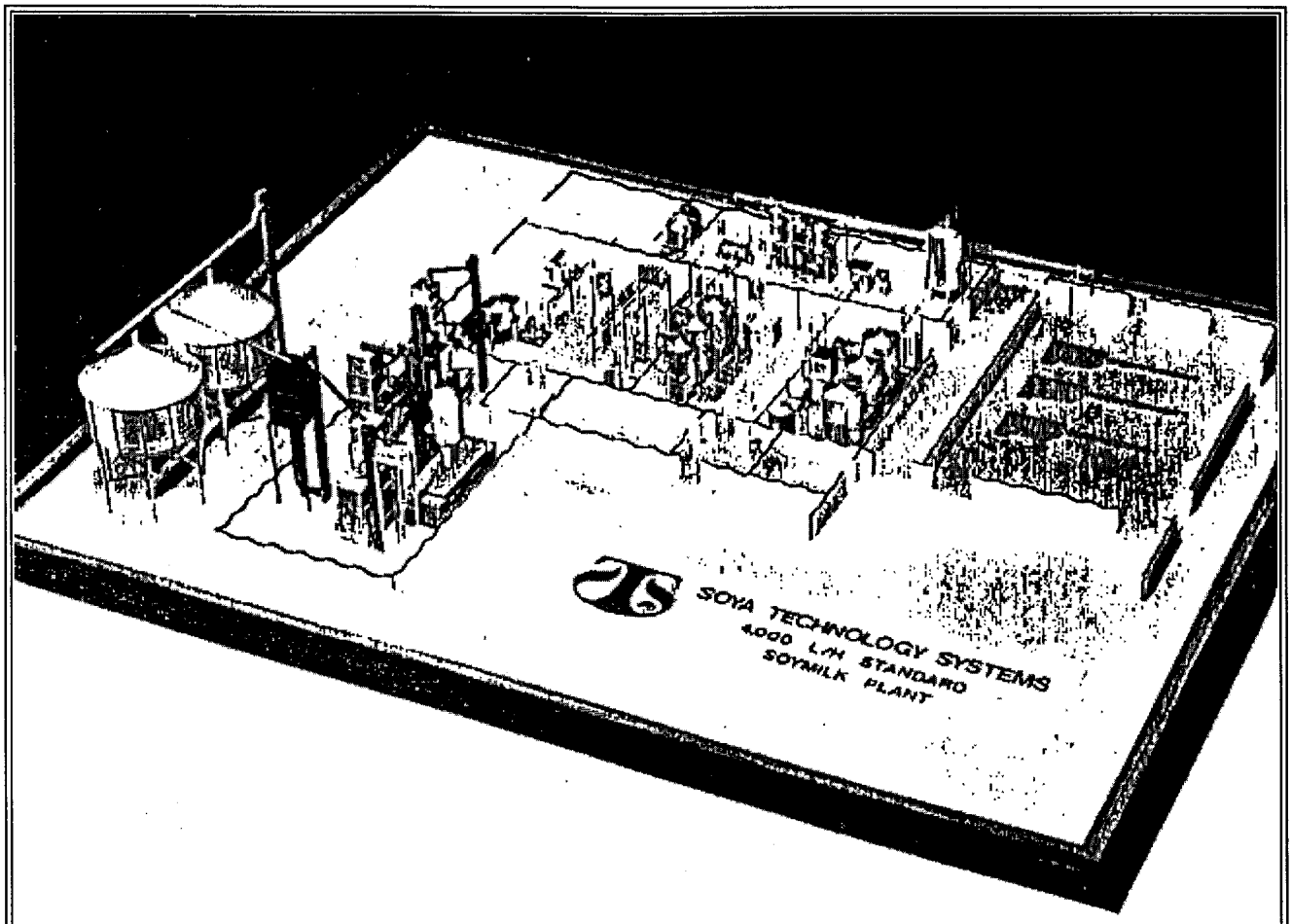


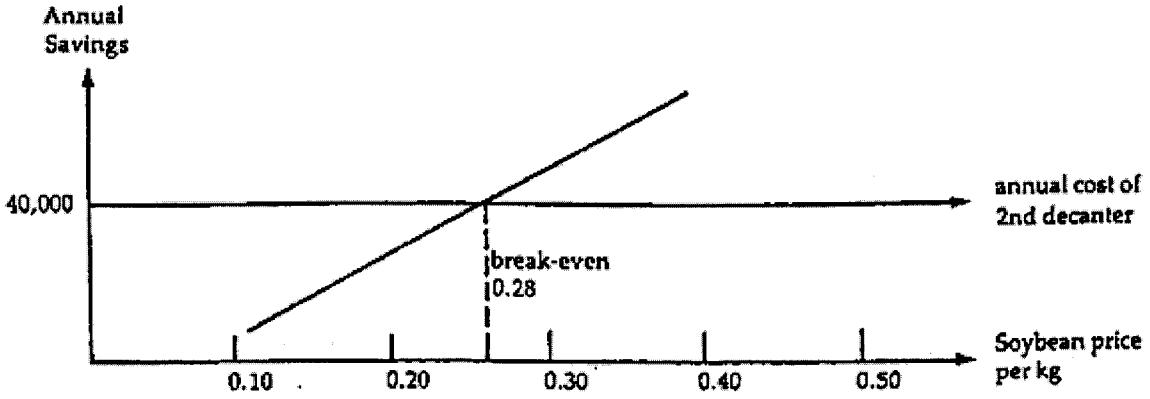
Figure 37: Economic Feasibility of Adding a Second Decanter to Soymilk Production Line (Courtesy: Danish Turnkey Dairies Ltd.)

The "dairy type" approach is widely applied in Japan. It has been reported that more than 75% of the soymilk in Japan is UHT processed and aseptically packaged. The price of soymilk in Japan is 25% higher than that of cow's milk.

As in all water extraction based processes for the production of soy milk, the STS process generates a by-product, namely the insoluble extraction residue or "pulp". This material comes out of the solids discharge of the decanter and contains the insoluble proteins and fibre as well as an entrapped portion of the extract. The yield of soy milk can be increased by providing an additional stage for the extraction of the residue with water, followed by an additional stage of separation in a second decanter. The economic feasibility of this second stage depend on the cost of a decanter against the price of soybeans. The determination of the break-even point is illustrated in Fig. 38. The pulp is not a waste but a useful by-product which can be sold as a feedstuff or a food ingredient with a high dietary fibre content and a good protein concentration.

The distribution of protein between the product and the residue is shown in Fig. 39. (In this diagram, the residue is called by its Japanese name: "**Okara**".)

Annual savings in purchase of soybeans by using a second decanter



ASSUME: Plant capacity : 4000 L/H (3.5% protein)
soybeans saved/hour : 40 kg
soybeans saved/year : 120,000 kg
(10 hour/day, 300 days/year)

Annual cost of a decanter
depreciation : 40,000
variable costs

This example shows that if the price of soybeans is below 0.28/kg, a second decanter is not an economical proposal but that money could be saved if the price of soybeans is higher than 0.28/kg.

Figure 38: Economic Feasibility of Adding a Second Decanter to Soymilk Production Line
(Courtesy: Danish Turnkey Dairies Ltd.)

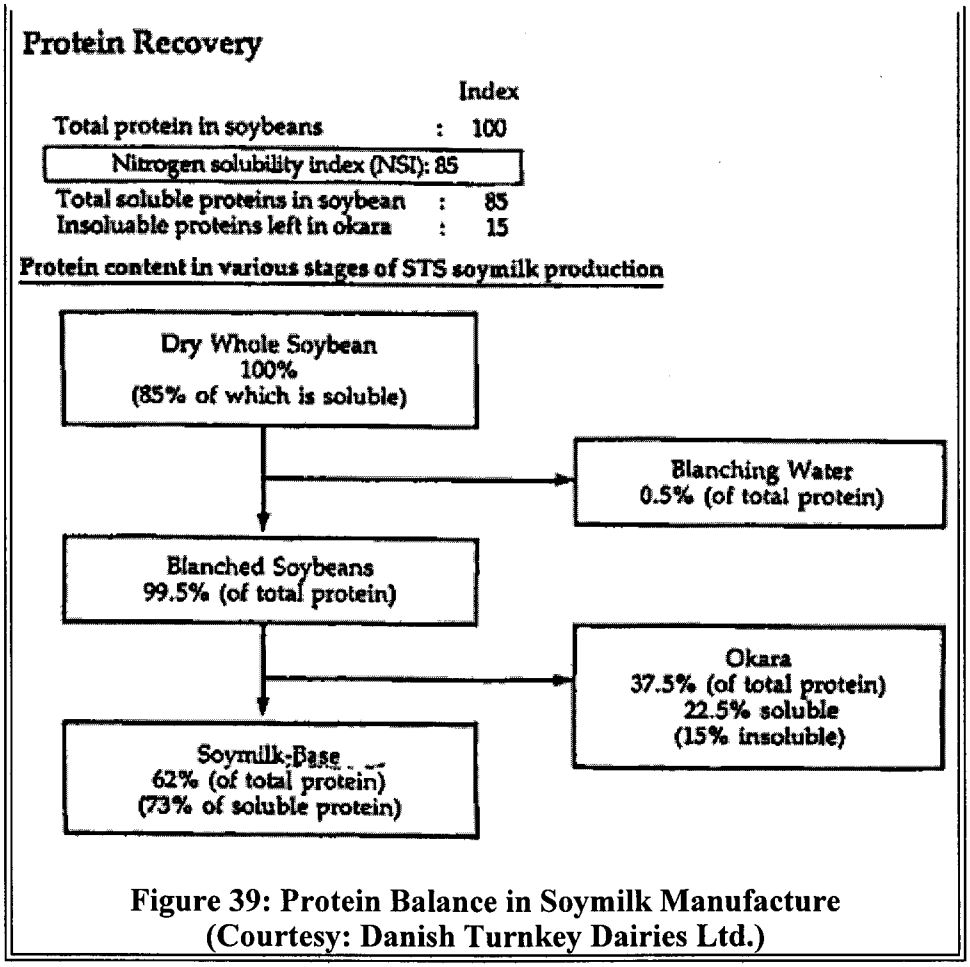


Figure 39: Protein Balance in Soymilk Manufacture
(Courtesy: Danish Turnkey Dairies Ltd.)

8-3-3 The INTSOY (Illinois) process

The development of this process resulted from research done at the University of Illinois at Urbana-Champaign in the early 1970s and was picked up later by INTSOY (International Soybean Program). INTSOY is a program supported by U.S. and international agencies and managed by the University.

The "**Illinois process**" is basically similar to the STS process described above. Actually, the basic process of lipoxidase inactivation by blanching the whole beans in a hot dilute solution of bicarbonate originated in Illinois. The soybeans are cleaned, sized, oven dried by forced air, split and dehulled. The cotyledons are blanched in boiling water containing sodium bicarbonate, drained and ground in boiling water . After thorough mixing, the slurry is strained. The filtrate (milk) is heated, homogenized, pasteurized and packaged.

In contrast to the STS Process,the INTSOY process has been designed for small-scale plants. These plants are sold by BAR Export/Import, Inc. a company based in Urbana, Illinois. A price quotation for a complete plant for 220 litres of soymilk per hour is appended. (See Annexes).

8-3-4 The BUHLER Process for Soy Micro-powder

Buhler Ltd. from Switzerland presents its patented process for the manufacture of dehulled, micro-milled, full-fat soy flour as an alternative or as a supplement to conventional soymilk production. Two features distinguish the process from ordinary full-fat soy flour manufacturing : the hot dehulling process and micromilling of the cotyledon particles to an impalpable powder by means of a six-roller

mill. Hot dehulling, mentioned and described in Chapter 3, is said to reduce bitterness. Micromilling is claimed to break the cells, liberating the protein bodies and fat globules and making them more available for extraction.

A flow diagram of the Buhler Process is shown in Fig. 40.

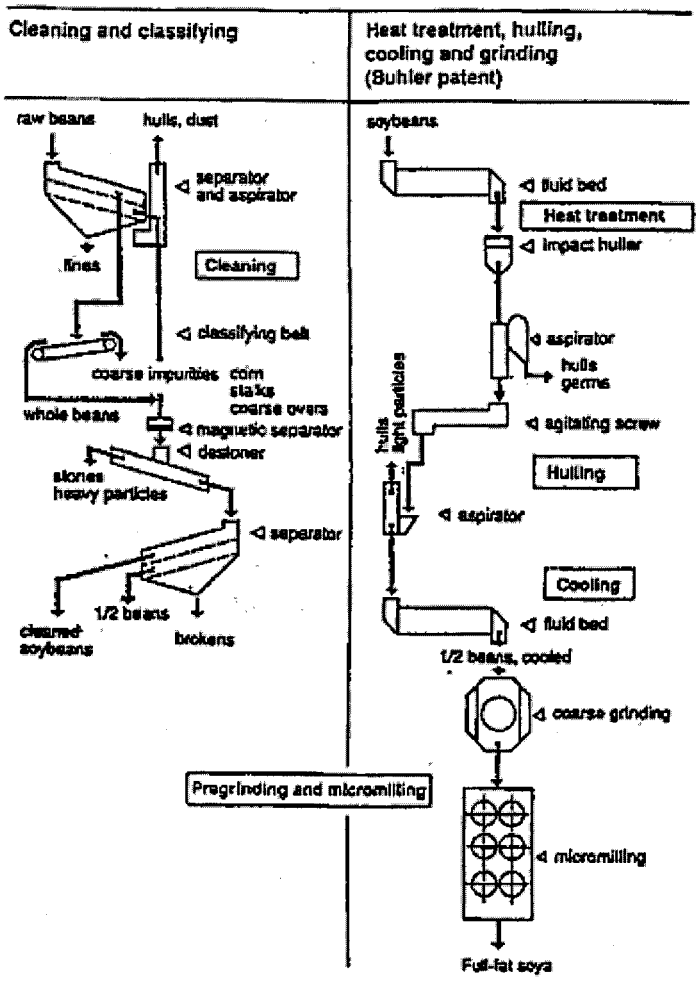


Figure 40:

The BUHLER Process For Micromilled Soya Powder
Source: Gawin and Wettstein (1990)

According to Buhler publications, the fine powder is easily dispersed in beverages such as chocolate drinks and can replace milk solids in such products. A suspension of the micro-pulverized full-fat soy powder in water is referred to as "soymilk", although it has all the fibre of dehulled soybeans. If a fibre-free normal soymilk is desired, the micro-milled flour can serve as the starting material . It is claimed that the yield of soymilk per unit weight of soybeans is almost doubled, due to the fact that micro-milling releases the proteins and fats, rendering their extraction more complete. The residue (okara) now consists of a relatively small amount of cell-wall debris, its quantity being only 1/8 of that of okara in the traditional extraction process. (Fig. 41).

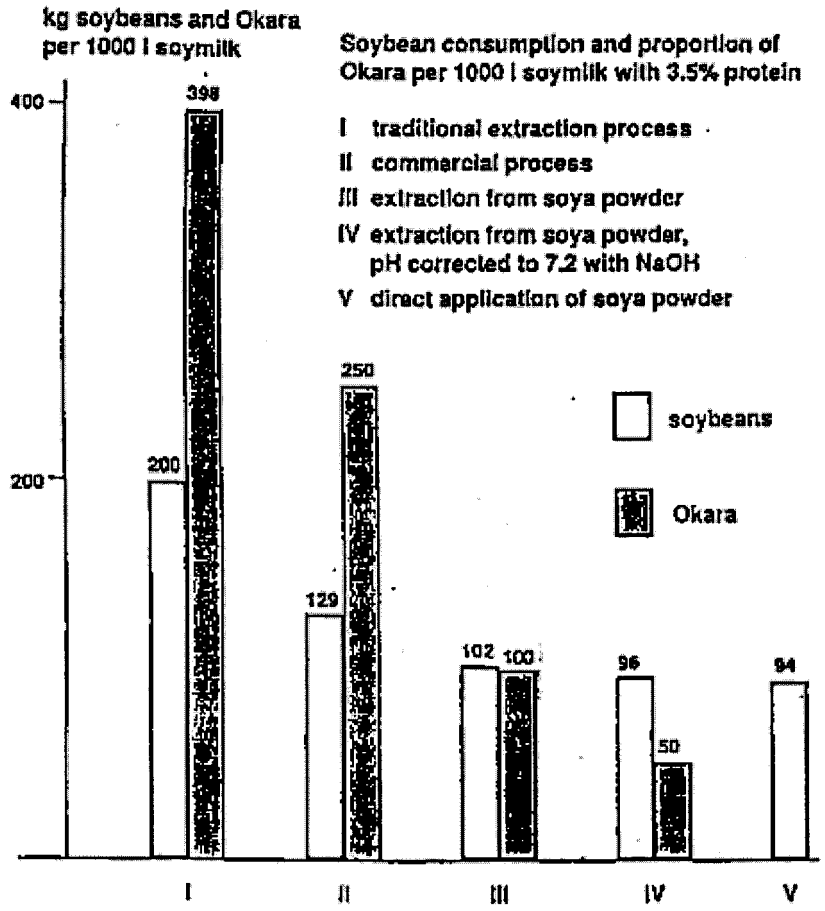


Figure 41: Soybean Consumption and Quantity of Residue Produced per 1000 litre of Soymilk,
in various processes Source: Gawin and Wettistein (1990)

Vol. 179, No. 1, 1991
August 30, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 661-667

GENISTEIN INHIBITION OF THE GROWTH OF HUMAN BREAST CANCER CELLS: INDEPENDENCE FROM ESTROGEN RECEPTORS AND THE MULTI-DRUG RESISTANCE GENE

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Received July 25, 1991

The effect of isoflavones on the growth of the human breast carcinoma cell lines, MDA-468 (estrogen receptor negative), and MCF-7 and MCF-7-D-40 (estrogen receptor positive), has been examined. Genistein is a potent inhibitor of the growth of each cell line (IC_{50} values from 6.5 to 12.0 μg/ml), whereas biochanin A and daidzein are weaker growth inhibitors (IC_{50} values from 20 to 34 μg/ml). The isoflavone β-glucosides, genistin and daidzin, have little effect on growth (IC_{50} values >100 μg/ml). The presence of the estrogen receptor is not required for the isoflavones to inhibit tumor cell growth (MDA-468 vs MCF-7 cells). In addition, the effects of genistein and biochanin A are not attenuated by overexpression of the multi-drug resistance gene product (MCF-7-D40 vs MCF-7 cells).

Rats consuming a soy-based diet develop a lower number of mammary tumors following administration of the carcinogens N-methylnitrosourea and 7,12-dimethylbenz[a]anthracene than rats on isonitrogenous and isocaloric diets without soy (1). We have speculated (1) that the aglucones of the isoflavones in soy, genistein (5,7,4'-trihydroxyisoflavone) and daidzein (7,4'-

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Abbreviations: ER, estrogen receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MDR, multi-drug resistance; TPK, tyrosine protein kinase; EGF-R, epidermal growth factor receptor; HPLC, high performance liquid chromotography; gp 170, a 170,000 Da glycoprotein, a product of the multidrug resistance gene.

0006-291X/91 $1.50

dihydroxyisoflavone), may have properties similar to the antiestrogen drug tamoxifen, which competes with estrogen for occupancy of the estrogen receptor (ER), thereby inhibiting the metastatic growth of breast cancer. On the other hand, it has been shown that isoflavones, particularly genistein, are potent inhibitors of the tyrosine protein kinase (TPK) activity of growth factor receptors, such as epidermal growth factor receptor (EGF-R) (2), and several oncogenes which may be associated with tumor cell growth and tumor recurrence, such Ha-ras (3) and $pp56^{lck}$, a src-family kinase (4). Since many recurrent breast cancers are ER-independent, a drug or dietary agent that inhibits the growth of both ER^+ and ER^- tumors would be of great interest.

In addition, it is important to determine whether such compounds are substrates of the multi-drug resistance (MDR) gene product, P-glycoprotein. This 170 KDa cell membrane protein (gp 170) confers resistance to a wide range of chemotherapeutic agents by acting as a drug efflux pump, thereby reducing the concentration of the drug in the cytoplasm of the tumor cell (5).

In this study, we have examined whether: (1) soy isoflavones inhibit the growth of human breast cancer cells in culture, (2) whether inhibition is dependent on the expression of the ER and (3) whether inhibition is attenuated by expression of gp 170.

MATERIALS AND METHODS

Materials: Soy molasses was a gift of the Archer Daniels Midland Co. (Decatur, IL). Fetal bovine serum and antibiotics were obtained from Gibco (Gaithersburg, MD). Tissue culture supplies were from Costar (Charlotte, NC). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), doxorubicin, α- and β-glucosidases and biochanin A (4'-methoxygenistein) were from Sigma Chem. Co. (St. Louis, MO). Microtiter plates, Sephadex G-25 and CNBr-activated Sepharose were purchased from Pharmacia (Piscataway, NJ). Aquapore C_8 columns were from Brownlee Labs (Santa Clara, CA).

Cell Culture: MCF-7 and MCF-7 D-40, and MDA-468 human breast cancer cells lines were gifts of Dr. William Dalton (University of Arizona) and Dr. Jeff Kudlow (Division of Endocrinology, University of Alabama at Birmingham), respectively. MCF-7 and MCF-7 D-40 cells were maintained in RPMI 1640 medium supplemented with 7% (v/v) fetal-bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin); MCF-7-D-40 cells also received 10^{-8} M doxorubicin to maintain the MDR phenotype; MDA-468 cells were maintained on Dulbecco's Modified Eagles medium low glucose, with 10% (v/v) fetal bovine serum and antibiotics (as above). Cells were cultured as

monolayers (passed every 7-8 days) in a 95% air: 5% CO_2, water-saturated atmosphere.

Isoflavone Preparation: Genistin and daidzin were isolated from soy molasses by fractional crystallization (6) and by adsorption chromatography (7), respectively. Their aglucones, genistein and daidzein, were prepared by hydrolysis in methanol: 1 M HCl (1:1 v/v).

Sample Preparation: Isoflavone samples were prepared from 10, 5, or 2.5 mg/ml stock solutions in DMSO. Aliquots were then taken to prepare the various samples (final concentrations from 1 to 100 μg/ml). DMSO was added as necessary to give a final DMSO concentration of 1% (v/v) in each well.

HPLC Analysis: The purity of the stock solutions and the stability of the isoflavones in the tissue culture media during incubation with the cells were determined by reversed-phase HPLC on a 30 x 0.45 cm Aquapore C_8 column using gradient elution with a mobile phase consisting of 0-45% acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid. Eluting substances were detected by their absorbance at 262 nm.

Viability Assay: Cytoxicity of the isoflavones was determined by a modification of the MTT assay (8), which is based on the reduction of MTT by the mitochondrial dehydrogenases of viable cells. Cells were plated into 96-well tissue culture clusters at densities of 2 x 10^3 cells/well (MCF-7), 10^4 cells/well (MCF-7-D40) and 2.5 x 10^3 cells/well (MDA-468) in 198 μl of media (optimal numbers of cells for each well were previously determined by ^3H-thymidine uptake). After plating, the cells were allowed to attach for 2 days. Isoflavones were then added (2 μl volumes as described) and incubation continued for 4 days; control wells received 2 μl DMSO. After 4 days, 50 μl of 2 mg/ml MTT was added to each well and the plates incubated for 4 h at 37° C. Media and unreacted MTT were then removed by gentle aspiration. One row of cells had the media removed for HPLC analysis. DMSO (100 μl) was added to each well and the plates were gently shaken for 5 min at room temperature. The optical density at 540 nm was immediately determined using a MAXLINE plate reader (Molecular Devices, Menlo Park, CA). Absorbance at 690 nm was also measured to compensate for interfering effects of cell debris and the plate itself. The percent survival was determined by comparing the absorbance for treated cells to that obtained for control cells. Each experiment consisted of 3 plates, and the results given are the mean and standard error of three separate experiments.

RESULTS AND DISCUSSION

Genistein was a potent growth inhibitor in both MCF-7 cells (IC_{50} 10.5 μg/ml) and MDA-468 cells (IC_{50} 6.5 μg/ml) (Fig. 1A). Biochanin A had weaker inhibitory effects on the growth of MCF-7 and MDA-468 cells (IC_{50} values of 22 μg/ml and 30 μg/ml, respectively) (Fig 1B). Daidzein, also had weak effects on cell growth, with IC_{50} values of 28 μg/ml for MCF-7 cells and 34 μg/ml for MDA-468 cells (Fig 1C). The isoflavone β-glucosides, genistin and daidzin, were not effective

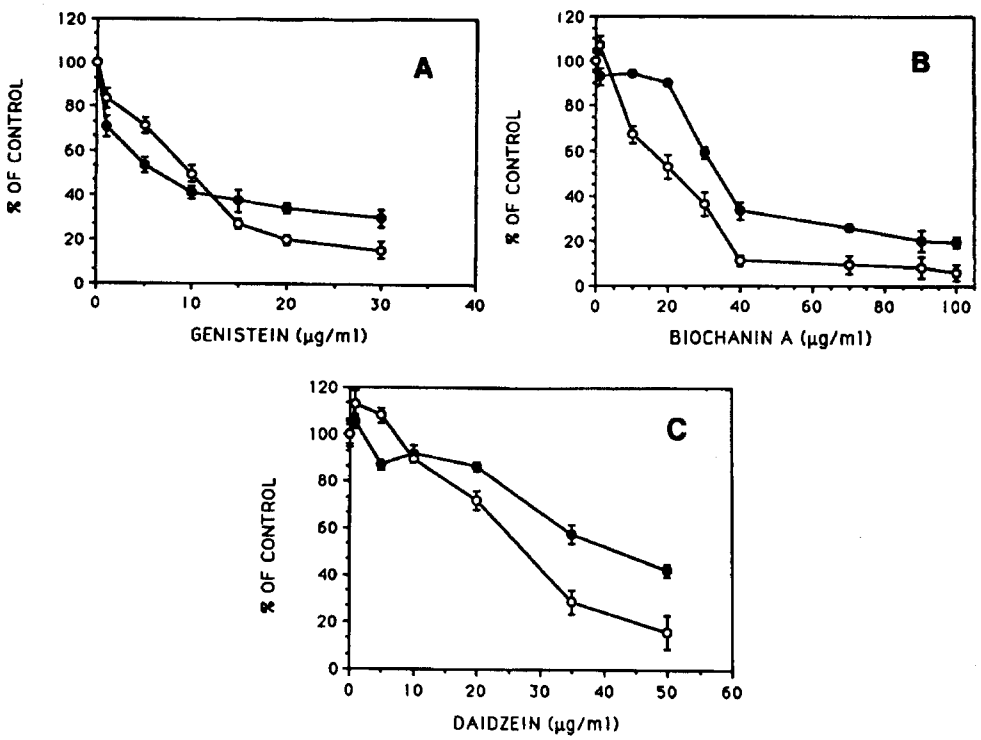

Figure 1. Inhibition of growth relative to controls of MCF-7 (0) and MDA-468 (●) cells by the isoflavones genistein (panel A), biochanin A (panel B) and daidzein (panel C).

in inhibiting cell proliferation, with IC_{50} values above 100 µg/ml (Fig. 2A and B). The weak growth inhibition observed at higher concentrations of genistin in MDA-468 cells appeared to be due to tumor-cell induced hydrolysis of genistin to genistein (data not shown). This hydrolysis did not occur in MCF-7 cells.

There was no significant difference in the potency of growth inhibition of MDA-468 and MCF-7 cells by each isoflavone. These data suggest that the

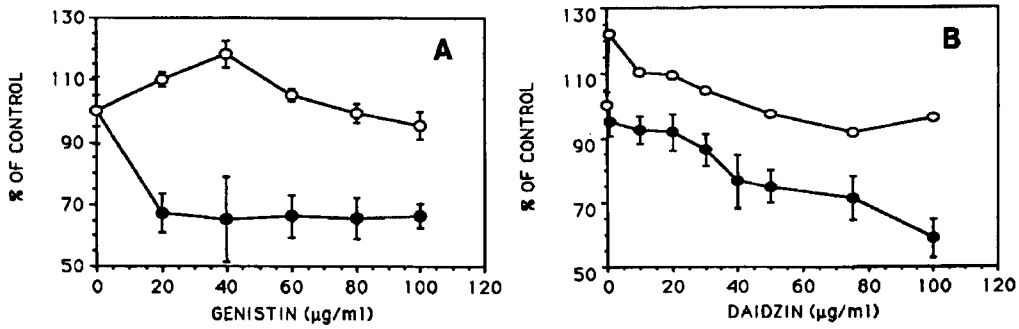

Figure 2. Inhibition of growth relative to controls of MCF-7 (O) and MDA-468 (●) cells by the isoflavones genistin (panel A) and daidzin (panel B).

isoflavones can act via an ER-independent pathway. This does not, however, rule out the involvement of ER in isoflavone action in ER^+ cells. Recent evidence has shown that increased phosphorylation of the estrogen and progesterone receptors can alter the activity of these receptors (9,10). The isoflavones could, therefore, in part, exert their effect by interfering with their phosphorylation state.

The precise mechanism of action of genistein, and of isoflavones in general, on tumor cell proliferation is at present unknown. The effect of genistein is not, however, non-specific; although the growth of ras-transformed NIH-3T3 cells was inhibited by genistein, the growth of non-transformed cells at the same genistein concentration was unaffected (11). Genistein inhibits the intrinsic TPK activity of many growth factor receptors, including EGF-R (2,3) and platelet-derived growth factor receptor (12). The isoflavones could also inhibit targets downstream of the activated receptor such as phospholipase C-γ, phosphatidylinositol kinases, or MAP kinase, all of which show increased tyrosine phosphorylation in response to EGF treatment (13,14,15,16). In support of this view, the isoflavone psi-tectorigenin (8-methoxygenistein) has been shown to inhibit cellular phosphatidylinositol turnover without inhibiting EGF-R TPK activity in A431 fibroblasts (17). Also, genistein can cause cytostatic effects on cell growth without inhibiting the EGF-R TPK activity in NIH-3T3 cells, possibly due to its preferential inhibition of ribosomal S6 phosphorylation (18), which is thought to occur via MAP kinase (19). However, Ogawara et al. (3) found no close correlation between inhibition of EGF-R tyrosine kinase activity in vitro and the reduction in the growth of Ha-ras transformed NIH-3T3 cells.

An alternative mechanism for the action of isoflavones is their inhibition of DNA topoisomerases. Genistein has been shown to inhibit mammalian DNA topoisomerase II in L-1210 cells (12). Also, a chinese hamster ovary cell line with altered DNA topoisomerase activity has been isolated that is more resistant to genistein than the parental cell line (20).

A derivative of the MCF-7 cell line, MCF-7-D40, which overexpresses gp 170, is resistant to the potent anticancer drug doxorubicin (Fig. 3A) (6). However, the

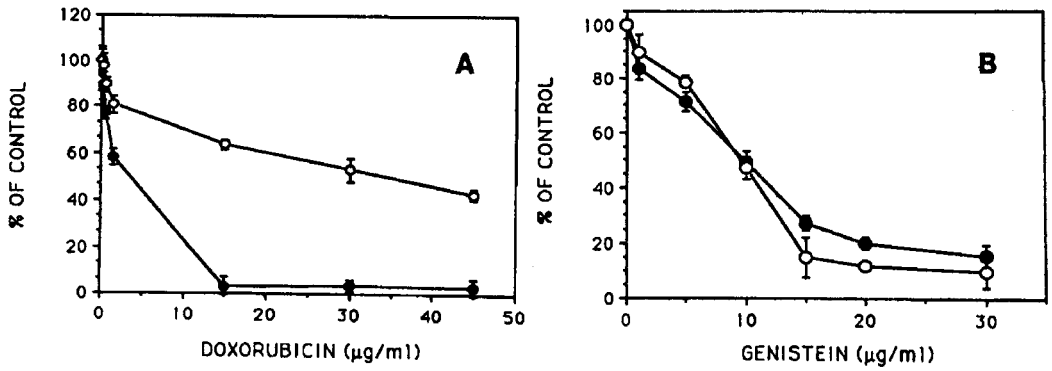

Figure 3. Inhibition of growth of MCF-7 wild type (●) and MCF-7-D40 (○) cells by doxorubicin (panel A) and genistein (panel B).

IC_{50} for genistein was the same for the MCF-7 and MCF-7-D40 cell lines (Fig. 3B). In addition, the IC_{50} for biochanin A was lower in the MCF-7-D40 cells than in the MCF-7 cells (data not shown). These results show that neither genistein nor biochanin A are adversely effected by overexpression of gp 170, and suggest that the isoflavones, in general, may be immune to the multidrug resistance phenomena. In support of this observation, Honma et al. have shown that genistein induces differentiation of a multi-drug resistant K562 (human myelogenous leukemia) cell line as effectively as in its parental cell line (21).

The data obtained in this study support the notion that the isoflavones genistein and daidzein are active anti-cancer agents in soy. Thus soy, a significant part of the diet of many Orientals, may be an important factor which accounts for the low rate of breast cancer in Oriental women (22).

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[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, PURDUE UNIVERSITY AGRICULTURAL EXPERIMENT STATION, IN COÖPERATION WITH U. S. REGIONAL SOYBEAN INDUSTRIAL PRODUCTS LABORATORY][1]

Genistin (an Isoflavone Glucoside) and its Aglucone, Genistein, from Soybeans

BY E. D. WALTER

Genistein, which is now known to occur in soybeans as the aglucone of genistin, was isolated from Dyer's Broom (*Genista tinctoria*) in 1899 by Perkin and Newbury.[2] The isoflavone nucleus was established for genistein by Baker and Robinson[3] in 1926, when they found it to be identical with prunetol, and in 1928 they synthesized genistein. The constitution of genistein was thereby established as 5,7,4′-trihydroxyisoflavone.

In 1931 Walz[4] isolated genistin from a 90% methanol extract of soybean meal. He found that hydrolysis of genistin with hydrochloric acid gave one mole of genistein and one mole of glucose. The glucose was identified by optical rotation and by a Bertrand determination of the reducing sugar after its separation from the aglucone. From degradative experiments he found that the glucose was bound to genistein at position seven and that the constitution of genistin was 5,7,4′-trihydroxyisoflavone-7-glucoside.

In 1939 Okano and Beppu[5] reported four isoflavones from soybeans. One of these was named isogenistein, but apparently none of them had the same physical properties shown by genistin or genistein.

During the course of a study of the carbohydrates of soybeans, an attempt was made to isolate certain sugars from a methanol extract of oil-free soybean meal. After the removal of some of the phosphatides from a concentrated methanol solution by the addition of acetone, the solution was concentrated and upon the addition of water, white, granular aggregates crystallized. These crystals, when dissolved in hot ethanol, gave a reddish-violet coloration with an ethanol solution of ferric chloride. The properties of the purified material agreed with those reported by Walz for genistin.

This paper confirms some of Walz's work and presents further evidence that the sugar hydrolyzed from genistin is *d*-glucose. Additional physical and chemical properties of genistin and its aglucone, genistein, are also presented. The melting points, optical rotation and carbon and hydrogen analyses of the compounds presented are in agreement with the data presented by Walz for the corresponding substances.

A small amount of some other crystalline material was obtained from the mother liquors, which gave qualitative evidence for another flavone. Work is in progress in this Laboratory on the isolation and identification of this material.

Experimental

Isolation and Properties of Genistin.—Commercial soybean flakes, which had been extracted with hexane, were placed in a cloth bag in 10-kg. quantities and put into the extraction chamber of a Lloyds extractor. Sufficient methanol (about 24 liters) was added to cover the bag of flakes. The valves in the apparatus were so adjusted that the extractor would operate as a reflux. Steam was passed through the coil surrounding the extraction chamber and the mixture was refluxed for twenty-four hours. The extract was drained off, a fresh supply of methanol was added to the flakes and the mixture was again refluxed for twenty-four hours. The extracts were combined and concentrated to a volume of about 1.5 liters. Acetone was added to the concentrate until precipitation ceased. The acetone precipitated some of the phosphatides, which carried with them carbohydrates, saponin and other impurities. After the supernatant liquid became clear, it was decanted and concentrated on the steam-bath to a thin sirup. About two volumes of water was added to this sirup and on standing for a day at room temperature the genistin crystallized in yellowish-white granular aggregates.

The crude genistin was removed from the mother liquor by centrifuging. It was then dissolved in approximately 80% hot ethanol, treated with Nuchar and filtered. Crystallization was effected by concentrating the filtrate and cooling it to room temperature. At this point the crystals were contaminated with saponin. This was detected readily by adding a few crystals to concentrated sulfuric acid. When saponin is present the citron-yellow color due to genistin soon changes to red and later to purple. With pure genistin, however, the yellow color with sulfuric acid is permanent. After several recrystallizations from 80% ethanol the genistin was obtained in pale-yellow thin rectangular plates of m. p. 256° (Walz recorded 254–256°). Further treatment of the mother liquors with Nuchar, followed by concentration, yielded additional genistin. The total yield was 0.10% of the dry hexane-extracted soybean flakes.

(1) The U. S. Regional Soybean Industrial Products Laboratory is a coöperative organization participated in by the Bureaus of Agricultural Chemistry and Engineering and Plant Industry of the United States Department of Agriculture and the Agricultural Experiment Stations of the North Central States of Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota and Wisconsin.

(2) A. G. Perkin and F. G. Newbury, *J. Chem. Soc.*, **75**, 830 (1899).

(3) W. Baker and R. Robinson, *ibid.*, 2713 (1926); 3115 (1928).

(4) E. Walz, *Ann.*, **489**, 118 (1931).

(5) K. Okano and I. Beppu, *Bull. Agr. Chem. Soc. Japan*, **15**, 110 (1939).

When a few drops of ferric chloride was added to an ethanol solution of the substance, a reddish-violet coloration was obtained. The substance was insoluble in cold water, but slightly soluble in hot water, hot ethanol and in hot methanol. It was soluble in hot 80% ethanol, hot 80% methanol and in hot acetone. It was quite soluble in pyridine. When dissolved in alkali it gave a yellow color.

Genistin dried in an Abderhalden drier at 110° gave the following analytical data—calcd. for $C_{21}H_{20}O_{10}$: C, 58.31; H, 4.66. Found: C, 58.05; H, 4.70. *Optical rotation.* 0.3 g. in 50 ml. of *N*/50 NaOH at 21° rotated −0.336°; l = 2.0 dm.; $[\alpha]^{21}D$ −28°. Walz recorded for genistin $[\alpha]^{21}D$ −27.7°.

Genistin (1.0 g.) was acetylated by refluxing for one hour with acetic anhydride (20 ml.) and one drop of concentrated sulfuric acid. The product was separated from the reaction mixture by the addition of water and filtration of the precipitate. The substance crystallized from ethanol in long prisms with a tendency to twist into spirals; yield 1.5 g., m. p. 188°. Walz reported for genistin hexaacetate a melting point of 188°.

Anal. Calcd. for $C_{21}H_{14}O_{10}(CH_3CO)_6$: C, 57.87; H. 4.71; CH_3CO, 37.73; mol. wt., 684. Found: C, 57.78; H, 4.92; CH_3CO, 37.63; mol. wt. (Rast), 686.

The acetyl determinations reported herein were made by the Kunz[6] method as follows. To 100 mg. of the substance 25 ml. of acetone was added. The mixture was cooled in an ice–salt-bath and 25 ml. of 0.1 *N* NaOH was added dropwise. The solution was then kept below 0° for one hour and back-titrated with 0.1 *N* hydrochloric acid. A blank was run on the acetone used.

Isolation of the Aglucone, Genistein.—Genistin (4 g.) was treated with 90 ml. of methanol and 20 ml. of concentrated hydrochloric acid. After about three hours of refluxing the substance was all in solution, but hydrolysis was continued for another three hours. Water was then added to the cooled solution to precipitate the aglucone. Ether was added to dissolve the precipitate and the two layers were separated by means of a separatory funnel. The ether was evaporated and the substance was crystallized from hot 60% ethanol, as white, rectangular and six-sided rods; yield 2.3 g., m. p. 296° (dec.). Walz recorded a melting point of 296–298° for genistein. The substance was water-insoluble, but was soluble in the usual organic solvents. It gave a reddish-violet coloration with ferric chloride. For analysis the substance was dried in an Abderhalden drier at 110°. The water solution from the above reaction mixture was saved for the identification of the sugar.

Anal. Calcd. for $C_{15}H_{10}O_5$: C, 66.6; H, 3.73; mol. wt., 270. Found: C, 66.66; H, 3.60; mol. wt. (Rast), 282. *Optical rotation.* 0.1029 g. in 25 ml. of acetone gave no rotation.

Genistein (1 g.) was acetylated by refluxing for one hour with 20 ml. of acetic anhydride and a drop of concentrated sulfuric acid. The mixture was poured into ice water and the precipitate was filtered off. The product crystallized from ethanol in white, needle clusters and an occasional boat-shaped form; yield 1.05 g., m. p. 200°. Walz recorded a melting point of 200–202° for genistein triacetate. It was soluble in organic solvents.

Anal. Calcd. for $C_{15}H_7O_5(CH_3CO)_3$: C, 63.63; H, 4.07; CH_3CO, 32.58; mol. wt., 296. Found: C, 63.16; H, 4.18; CH_3CO, 32.17; mol. wt. (Rast), 293.

Color Tests.—The reduction color tests described by Asahina and co-workers[7] as characteristic of flavones were also given by genistein and genistin.

The reactions were performed according to the directions of Wolfrom and co-workers,[8] who applied them to osage orange pigments. Reduction with magnesium and hydrochloric acid gave no coloration with either genistin or genistein. Reduction with sodium amalgam, however, produced orange-red solutions forming dark red precipitates on acidification with an excess of concentrated hydrochloric acid.

Both genistin and genistein gave a yellow coloration with the boric acid test, which was carried out as described by Wilson.[9]

Identification of the Sugar as *d*-Glucose.—The aqueous hydrolyzate from genistin was heated on the steam-bath to evaporate most of the methanol, then refluxed for three hours to hydrolyze the methyl glucoside. The mixture was then neutralized with silver carbonate and clarified with Nuchar. The clear liquid was concentrated under reduced pressure to about 4 ml. The sirup was dissolved in 20 ml. of methanol and used to prepare the potassium aldonate by the method described by Moore and Link.[10] A quantitative reduction on an aliquot of the liquid previous to neutralization showed a total of 0.796 g. of sugar calculated as glucose. This amount (less the aliquot used, 19.9 mg.) gave 0.81 g. of potassium aldonate, m. p. 180° (dec.), the melting point reported for potassium gluconate by Moore and Link. Corresponding amounts of reagents were used on this to prepare the benzimidazole derivative as described by Moore and Link.[10] After treatment of the reaction product three times with Nuchar, the crystalline product melted at 215° (dec.). A mixture of this with known *d*-glucobenzimidazole also melted at 215° (dec.).

Glucobenzimidazole Picrate.—The above *d*-glucobenzimidazole was used to make the picrate as described by Moore and Link.[10] The product melted at 207° (dec.). When mixed with known glucobenzimidazole picrate, the melting point was unchanged.

Crystallographic Optical Properties

Genistin.—Crystallized from 80% ethanol genistin appears, under the microscope, as thin rectangular plates (Fig. 1). *In parallel polarized light (crossed nicols)*: the birefringence is strong. The extinction is parallel and the elongation is positive. Refractive indices: n_α = 1.580 found across the elongated plates; n_β = indet.; n_γ = 1.650 found along the elongation, all ±0.003. No interference figures were observed in convergent polarized light (crossed nicols), which were suitable for establishing the β-value.

(6) A. Kunz and C. S. Hudson, THIS JOURNAL, **48**, 1982 (1926).

(7) Y. Asahina and M. Inubuse, *Ber.*, **61**, 1646 (1928); **64**, 1256 (1931); Y. Asahina, G. Nakagome and M. Inubuse, *ibid.*, **62**, 3016 (1929).

(8) M. L. Wolfrom, P. W. Morgan and F. L. Benton, THIS JOURNAL, **62**, 1484 (1940).

(9) C. W. Wilson, *ibid.*, **61**, 2303 (1939).

(10) Stanford Moore and Karl P. Link, *J. Biol. Chem.*, **133**, 293 (1940).

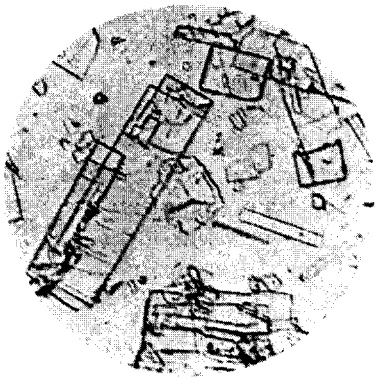

Fig. 1.—Genistin (× 75).

Genistein.[11]—*In ordinary light*, under the microscope, this substance shows broad, rectangular and six-sided rods, and hemimorph forms, pentagonal in outline (Fig. 2).

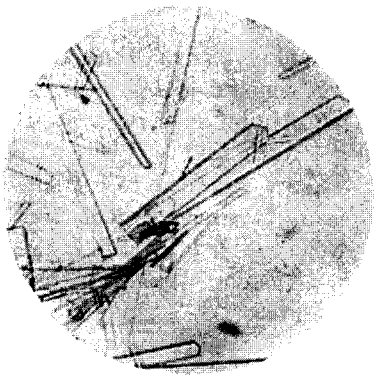

Fig. 2.—Genistein (× 75).

Refractive indices (determined by the immersion method in organic liquids): $n_\alpha = 1.530$; n_β = indet.; $n_i = 1.645$ (probably approximating the β-value); $n_\gamma = >1.733$ (methylene iodide), all ± 0.002, $n_\gamma - n_\alpha = 0.203$. n_α is frequently found lengthwise on rods, and on irregular fragments. No interference figures were observed in convergent polarized light (crossed nicols), therefore an intermediate index, $n_i = 1.645$ (commonly found), could not be positively confirmed as the β-value. n_γ is usually found on rods crosswise. *In parallel polarized light* (*crossed nicols*): the birefringence is strong and many of the rods show peculiar and characteristic blues or purples of the type frequently referred to as anomalous interference colors. The extinction is parallel and the elongation is negative.

Genistin Hexaacetate.—Crystallized from ethanol this substance appears in ordinary light as fine, long prisms. *In parallel polarized light* (*crossed nicols*) a peculiar barber-pole appearance is observed (Fig. 3). This is apparently due to the tendency of the slender crystals to twist. On the larger untwisted crystals the extinction is parallel and the elongation is positive. An occasional face shows no extinction and gives an optic axis interference figure in convergent polarized light (crossed nicols). On these $n_\beta = 1.556$. Across slender prisms showing red color between crossed nicols, $n_\alpha = 1.520$; $n_\gamma = 1.577$ found lengthwise, all ± 0.003.

Fig. 3.—Genistin hexaacetate under polarized light (× 540) (crossed nicols).

Genistein Triacetate.—This substance, when crystallized from ethanol, in ordinary light appears in clusters of needles and boat shaped forms (Fig. 4). *In parallel*

Fig. 4.—Genistein triacetate (× 75).

polarized light (*crossed nicols*): the birefringence is strong and many of the boat shaped crystals show characteristic blue and purple colors. The extinction is parallel and the elongation is negative. Refractive indices: $n_\alpha = 1.508$ is found lengthwise on the boat shaped forms; $n_\beta = 1.556$ is found on occasional fragments showing an optic eye (in convergent polarized light, crossed nicols); $n_\gamma > 1.733$ (methylene iodide) found crosswise on crystals showing blue color between crossed nicols, all ± 0.003.

Absorption Spectra of Genistin and Genistein.[12]—Figure 5 shows the ultraviolet absorption spectra of genistin and genistein in 85% ethanol. The ordinate is the specific absorption coefficient α, from the relation $\log I_0/I = \alpha cl$, with c the concentration in grams per liter and l the length of the cell in centimeters. The spectra were determined on a photoelectric spectrophotometer similar in principle to that described by Hogness and co-workers.[13]

(11) The writer is indebted to George L. Keenan, of The Federal Security Agency, Food and Drug Administration, Washington, D. C., for the optical crystallographic data for genistein reported here.

(12) The absorption spectra were determined by Jonathan W. White, Jr., of the Department of Agricultural Chemistry, Purdue University.

(13) T. R. Hogness, F. P. Zscheile, Jr., and A. E. Sidwell, Jr., *J. Phys. Chem.*, 41, 379 (1937).

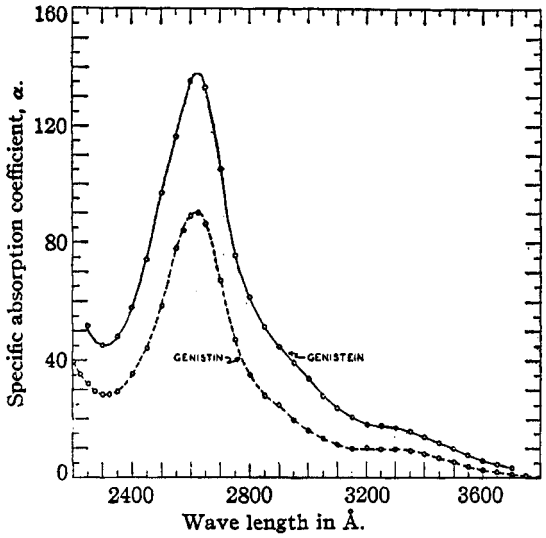

Fig. 5.—Absorption spectra of genistin and genistein.

A large Hilger double monochromator with quartz optics was employed to isolate spectral regions which varied between 6 and 13 Å. For genistin a single maximum was found at 2625 Å., with $\alpha = 90.5$. Recrystallization of the genistin did not affect the absorption spectrum. The absorption spectrum of the aglucone, genistein, was of the same general shape as that of genistin, but somewhat higher. The maximum occurred at 2625 Å., with $\alpha = 138$.

If one plots the absorption spectra data of Asahina and co-workers[14] for iridin, irigenin, tectoridin and tectorigenin in the same terms used in Fig. 5, a maximum is observed in the region of 2680 Å. for each of these compounds. A minimum occurs in the region of 2400 Å. and a shelf in the region of 3200 Å. Thus a similarity in the general shape of the curves exists between the absorption spectra of these compounds and those of genistin and genistein, although the maxima and minima do not occur at exactly the same wave lengths.

(14) Y. Asahina, B. Shibata and Z. Ogawa, *J. Pharm. Soc. Japan*, **48**, 150, 1093 (1928).

Summary

1. A crystalline pale yellow substance has been isolated from oil-free soybean meal. The data on this substance and its derivatives are in agreement with the data for genistin and its derivatives reported by Walz, who also isolated it from soybeans.

2. Additional evidence, that the sugar hydrolyzed from genistin is *d*-glucose, is presented.

3. The absorption spectra, a positive Wilson boric acid color reaction and a positive Asahina reduction test are recorded to further characterize genistin and its aglucone, genistein.

4. Crystallographic optical data are presented for genistin, genistein, genistin hexaacetate and genistin triacetate.

LAFAYETTE, INDIANA RECEIVED JULY 29, 1941

[CONTRIBUTION FROM THE FOOD AND DRUG ADMINISTRATION, FEDERAL SECURITY AGENCY]

Preparation of the Dinitrochlorobenzenes from the Corresponding Dinitroanilines*

BY LLEWELLYN H. WELSH

In connection with clinical studies of the specificity of response of persons eczematously sensitized to aromatic compounds, the need arose for specimens of the dinitrochlorobenzenes. Synthesis of five of the six possible isomers was required since only 2,4-dinitrochlorobenzene was commercially available.

The 3,4-,[1] 2,5-[2] and 2,6-[3] isomers are formed directly by the action of nitric acid on benzene derivatives. The last substance cited has also been prepared from tosyl chloride and 2,6-dinitrophenol.[4] Chlorination of *m*-dinitrobenzene has been reported to give 3,5-dinitrochlorobenzene.[5] The Sandmeyer reaction has been used in the preparation of 3,5-,[6] 2,5-,[7] 2,3-,[7a] 2,4-[8] and 2,6-dinitrochlorobenzene[9] from amino compounds.

For purposes of clinical study, the quantitative separation of the isomers was of more fundamental importance than the removal of unrelated impurities, and it was thought that the chances of satisfying this requirement would be increased by using syntheses which involved a plurality of steps between the isolation of the final product and the reaction which fixed the relationship of the substit-

* Not copyrighted.

(1) Laubenheimer, *Ber.*, **9**, 760 (1876); Mangini and Deliddo, *Gass. chim. ital.*, **63**, 612 (1933).

(2) Kehrmann and Grab, *Ann.*, **303**, 1 (1898).

(3) Borsche and Rantscheff, *ibid.*, **379**, 152 (1911).

(4) Kubota, *J. Chem. Soc. Japan*, **53**, 404 (1932); *C. A.*, **27**, 274 (1933).

(5) German Patent 108,165; *Chem. Zentr.*, **71**, I, 1115 (1900).

(6) Bader, *Ber.*, **24**, 1653 (1891); de Kock, *Rec. trav. chim.*, **20**, 111 (1901).

(7) (a) Hollemann and ter Weel, *ibid.*, **35**, 46–47 (1915); (b) Körner and Contardi, *Atti accad. Lincei*, **23**, I, 281 (1914).

(8) Hodgson and Walker, *J. Chem. Soc.*, 1620 (1933).

(9) Körner and Contardi, *Atti accad. Lincei*, **23**, II, 464 (1914).